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14. ABSTRACT
Work done during the first year of the award resulted in the identification and characterization of a set of 29 *A. baumannii* strains isolated from wounded military personnel with regard to their capacity to grow under iron-limiting conditions, produce iron chelators – siderophores – and form biofilms on abiotic surfaces found in medical settings. This analysis indicates that all isolates form biofilms on abiotic surfaces and different strains produce and/or use different siderophores when cultured under iron chelation. However, all tested strains produce dihydroxybenzoic acid derivatives, the synthesis of which could be used as a therapeutic target. Accordingly, salicylic acid derivatives seem to inhibit bacterial growth under iron chelation. Equally encouraging is the observation that gallium-containing derivatives, particularly Ga-protoporphyrin IX, inhibit bacterial growth independently of the iron and nutrient content of the medium. These are encouraging observations that will be tested in animal models proposed in this project and promote future basic studies since the mechanisms by which Ga-PPIX inhibit *A. baumannii* growth are unknown. Work done during this first year together with preliminary data collected by collaborators also resulted in the selection of the AB5075 isolate as a model strain for more extensive studies. This clinical isolate proved to be virulent when tested using *ex vivo* and *in vivo* experimental infection models and amenable to genetic manipulations, including high throughput insertion mutagenesis and genetic complementation with shuttle cloning vectors under construction.

15. SUBJECT TERMS
Wound infections, *Acinetobacter baumannii*, iron metabolism, biofilms

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Introduction

The overall purpose and scope of this research effort is to determine the role of iron acquisition and biofilm functions expressed by Gram-negative pathogens, particularly *Acinetobacter baumannii*, which cause severe infections in the Wounded Warrior because of polytrauma and blast injuries. The information collected with these studies will be used to explore the efficacy of different chemical and biological agents to block these potential virulence functions using appropriate experimental infection models. These studies have the potential of facilitating the development of new and more effective therapeutics to treat severe infections in wounded military personnel caused by Gram-negative bacteria.

Body

Aim 1. Examine the role of iron acquisition and biofilm formation functions expressed by Gram-negative bacteria in the pathogenesis of wound infections using animal models

Subaim 1.1. Select clinical isolates of A. baumannii for use as model strains in animal studies

Strain selection and typing

During the first year of the award we mainly focused on the study of the pathogen *Acinetobacter baumannii*. Strains from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN) at the Walter Reed Army Institute of Research (WRAIR) were classified by their isolation site, antibiotic resistance phenotype and pulse field gel electrophoresis (PFGE) characteristics. Standard clinical microbiology methods and PFGE techniques, which are based on the electrophoretic analysis of total DNA isolated from bacterial cells, were used for this purpose. This approach resulted in the identification of strains displaying unique PFGE patterns, which included strains obtained from different wound sites and expressing resistance to a wide range of antibiotics. From this collection of strains, 29 were selected for further studies (Table 1). Out of these 29 selected isolates, 25 represented 17 unique PFGE types while the PFGE type of five of them were not determined. All these isolates were obtained from different host sites or samples. We also included the clinical type strains 19606, 17978, and AYE, which we purchased from the American Type Culture Collection (ATCC), as well as the strain ACICU, which was provided to us by Dr. Daniel V. Zurawski from WRAIR. These four strains, which are not MRSN isolates, were used as controls since we have characterized their iron acquisition and biofilm formation properties in previous studies (1-3).

Iron acquisition and siderophore production/utilization phenotypes of selected strains

The iron acquisition phenotype of the selected clinical isolates listed in Table 1 was examined using Luria Bertani (LB) agar plates containing increasing concentrations of the synthetic iron chelator 2,2' dipyridyl (DIP), while the production and secretion of iron chelating compounds, known as siderophores, was tested using the Chrome Azurol S (CAS) colorimetric assay (4) and overnight culture supernatants of bacteria incubated in succinate minimal medium. These supernatants were also used to test the production of catechol-derived siderophores with the Arnow colorimetric reaction (5), using the 19606 and AYE strains as positive and negative controls, respectively (2). This analysis showed that all MRSN strains grew on LB agar plates under iron-chelated conditions imposed by the presence of DIP. However, not all strains have the same iron acquisition capacity. Strains 967, 2828, 3340, 3638, 3927, 4498, 4795, 5256, and 5674, although iron-uptake proficient, displayed a minimal inhibitory concentration (MIC) of DIP lower than 250 μM, while all the other MRSN strains tested were able to grow at this concentration of iron chelator. The four control strains also grew under the same experimental conditions. According to the CAS assay, which is used as a general reagent to detect siderophore production, all tested strains produce and secrete siderophores into the culture supernatant when grown in succinate minimal medium. Furthermore, the Arnow colorimetric assay, which detects the production of catechol-containing siderophores such as the acinetobactin siderophore produced by the ATCC 19606 type strain (6), showed that all isolates produce and secrete iron-regulated catechol compounds with the exception of the strain AYE. The lack of Arnow reaction of AYE culture supernatants is in accordance with our recently published data showing that this strain produces a non-catechol siderophore (2).

The iron acquisition phenotype of the MRSN isolates was also examined using biological assays designed to detect the production and/or utilization of acinetobactin. Siderophore utilization bioassays using the 19606-s1 *basD* mutant as a reporter strain, which does not produce but uses acinetobactin to grow under iron chelation (1), showed that cell-free iron-deficient culture supernatants from all strains, with the exception of isolate 3806, promoted the growth of this mutant in the presence of DIP (Table 1, *basD* assays). This result indicates that the strains produce and secrete active siderophore(s). Since the s1 *basD* mutant reporter strain might produce receptor proteins for siderophores different from acinetobactin, the secreted siderophores may or may not be related to acinetobactin. These possibilities were examined by testing the capacity of the MRSN strains to produce BauA, the outer membrane ferric acinetobactin receptor protein that plays a critical role in the ability of *A. baumannii* to grow under iron limitation and display virulence (7), and the production of acinetobactin with 19606 *bauA* mutant t6 bioassays. Proteins obtained from bacteria cultured under iron-limiting conditions were size-fractionated by SDS-polyacrylamide gel electrophoresis and probed with polyclonal antibodies against BauA as described before (1). The immunoblot assays (Table 1, BauA production) showed that all strains except isolates 3785, 3806 and 4498 produce BauA. Furthermore, bioassays using as a reporter strain the 19606 *bauA* mutant t6 (*bauA* assays), which produces acinetobactin but does not use it to grow under iron chelation because of a *bauA* insertion mutation (1), showed that cell-free iron-deficient culture supernatants from strains 967, 2828, 3638, 3785, 3927, 4025, 4269, 4448, 4456, 4490, 4932, 4957, 4991, 5001, 5075, 5256, 5674, and 5711 allow the *bauA* mutant to grow under iron chelation. In contrast, cell-free iron-deficient culture supernatants from strains 3340, 3560, 4026, 4027, 4052, 4498, 4795, 4857, 4878 and 5197 do not allow the *bauA* mutant to grow under iron chelation. Considering the results obtained with the *basD* and *bauA* assays together with the BauA production data, it is possible to conclude that strain(s):

1. 3785 could acquire iron via an acinetobactin-independent system since it does not produce BauA
2. 4498 could produce a catechol-derived siderophore different from acinetobactin that is not used by the s1 *basD* mutant and may represent a siderophore not produced by any of the MRSN tested isolates
3. 3340, 3560, 4026, 4027, 4052, 4795, 4857, 4878 and 5197 express only the acinetobactin-mediated iron acquisition system
4. 967, 2828, 3638, 3927, 4025, 4269, 4448, 4456, 4490, 4932, 4957, 4991, 5001, 5075, 5256, 5674 and 5711 could express iron acquisition functions mediated by acinetobactin and/or an unknown siderophore(s)

It is interesting to note that the ability of strain 3806, which has a lower MIC of DIP when compared with the other MRSN isolates, to produce detectable amounts of DHBA and catechol siderophores depends on the chemical composition of the media. Table 1 shows that when cultured in succinate minimal medium, this strain produces no detectable amounts of acinetobactin and/or DHBA when tested using colorimetric tests or biological assays. In contrast, the production of DHBA and acinetobactin is readily detectable in cell-free M9 minimal medium culture supernatants (Table 1, HPLC column). This is an interesting observation since we are not aware of reports describing such a drastic effect of the chemical composition, particularly the nature of the carbon source, of defined media normally used for siderophore production analysis. If time permits, we will study this phenomenon that could provide novel insights into the environmental factors that regulate the expression of bacterial iron acquisition systems.

The possibility that the MRSN strains produce different or more than one siderophore-mediated iron acquisition system was examined with reverse phase high-pressure liquid chromatography (RP-HPLC) of cell-free culture supernatants as we did for the analysis of the *A. baumannii* strains 19606 and AYE (2). Table 1 (HPLC column) and Fig. 1, profile 2, show, as expected from previous observations (2), that the AYE control strain produces neither acinetobactin nor its biosynthetic precursor dihydroxybenzoic acid (DHBA). Therefore, the ability of this strain to acquire iron depends on the production of a siderophore we predict is a hydroxamate rather than a catechol derivative. In contrast, 21 out of 29 (72%) MRSN strains produced an HPLC profile identical to that of 19606, which includes distinct peaks for DHBA and acinetobactin (Fig. 1, profile 1), while three others (3927, 4498 and 5197) make detectable amounts of DHBA but not acinetobactin (Fig. 1, profile 3). The latter observation together with the data collected using the bioassays shown in Table 1 suggest that these three strains may produce a DHBA-derived siderophore different from acinetobactin. The RP-HPLC analysis also showed that four strains (3560, 4456, 4857 and 4878) produce acinetobactin but no detectable amounts of DHBA (Fig. 1, profile 4), an outcome that could be due to differential expression of genes coding for DHBA and acinetobactin production. Finally, there is one strain, 4052, which produces acinetobactin as well as compounds with shorter retention times different from the signals produced by the sterile medium (Fig. 1, profile 5), an elution pattern similar to that of 17978. Interestingly, 17978 produces acinetobactin as well as a series of DHBA-based siderophore derivatives recently characterized as fimsbactins (8). Taken together, these results indicate that different *A. baumannii* MRSN clinical isolates acquire iron via different siderophore-mediated systems, although all of them seem to be DHBA derivatives. Therefore, targeting the production of acinetobactin and/or DHBA, which is a common precursor for the biosynthesis of catechol-based siderophores, is a reasonable approach to develop new strategies to treat infections caused by multi-drug resistant *A. baumannii* strains. It is also interesting to note that all these results and observations support our earlier report in which we hypothesized the ability of *A. baumannii* clinical isolates to produce and/or use different types of siderophores based only on: (1) the production of iron-regulated catechol compounds, (2) the production of the BauA acinetobactin outer membrane receptor protein, (3) the electrophoretic pattern of iron-regulated outer membrane proteins and (4) the responses of different clinical isolates when tested in siderophore utilization/production bioassays (9).

Outer membrane siderophore receptors as potential targets

Table 1 also shows that 26 out of 29 (almost 90%) of the MRSN strains produce the BauA acinetobactin outer membrane receptor, which most likely allows these strains to acquire iron during infection. The target value of this protein is further supported by the fact that 9 out of 29 (31%) MRSN strains utilize only the acinetobactin-mediated system to acquire iron under chelated conditions. On the other hand, the observation that 17 out of 29 (58%) MRSN strains could express the acinetobactin system together with an unknown siderophore-mediated system indicates that additional outer membrane siderophore receptors should be considered as potential targets to develop therapeutic antibodies to treat wound infections. Using these additional antibodies in concert with those directed toward BauA would be a more broad-spectrum therapeutic.

Based on these observations, we tested the reactivity of a selected group of MRSN strains with polyclonal antibodies raised using as antigen total membrane fractions isolated from *A. baumannii* 19606 and 17978 cells cultured under iron-limiting conditions. The objective was to develop antibodies that could be used to block functions such as ferric siderophore uptake, adherence to plastics and motility on semi-solid media. All these functions depend on the activity of cell-surface components such as outer membrane siderophore receptors, pili and outer membrane proteins that could be recognized by the human immune system. To test the value of this antiserum, we first confirmed the presence of antibodies against *A. baumannii* antigens by testing their reaction with whole-cell lysate proteins of strains 19606, 17978, 4857 and 5075 that were size-fractionated by gel electrophoresis and transferred to nitrocellulose filters. Figure 2A shows that the antibodies produced a strong reaction with a wide range of proteins present in all four strains cultured under iron-rich (LB) as well as iron-limiting (DIP) conditions. However, flow cytometry analysis, which tests the binding of anti-19606/17978 antibodies to the surface of intact bacterial cells, produced mixed results (Fig. 2B). As expected, because they were used to generate the tested antibodies, *A. baumannii* 19606 and 17978 cells produced clear signals showing the binding of anti-19606/17978 antibodies to bacteria cultured under iron-rich or iron-limiting conditions (right peaks on top panels of Fig. 2B). On the other hand, low signals were collected when cells of the strains 4857 and 5075 were tested under the same experimental conditions (middle and lower panels of Fig. 2B). These results could indicate that not all *A. baumannii* bacterial cells produce common surface antigens, an unlikely possibility considering the data obtained by immunoblotting (Fig. 2A), or that bacterial antigens are not readily accessible because of the presence of capsule, which could prevent the binding of antibodies to cell-surface proteins/structures. The latter possibility will be examined in more detail using proper staining and microscopy methods. We also plan to test the effect of these antibodies on the expression of cell functions such as motility and adhesion to abiotic surfaces during the second year of this award.

Inhibition of siderophore biosynthesis and iron utilization functions

Based on the data displayed in Table 1, which shows that all MRSN strains produce DHBA-derived compounds associated with iron acquisition under iron-limiting conditions, we initiated the testing of potential iron-uptake inhibitors using laboratory culture conditions. For this purpose, we explored the effect of salicylic acid derivatives on the iron uptake capacity of these clinical isolates since some of these derivatives inhibit the production of siderophores, particularly those derived from DHBA (10). For this purpose, the MRSN and control strains were cultured in M9 minimal medium containing DIP in the presence of different amounts of sodium salicylate, 5-chlorosalicylic acid, salicylamide, diflunisal, 5-sulfosalicylic acid, 2-hydroxy-5-nitrobenzoic acid, 5-aminosalicylic acid, and 4-aminosalicylic acid. Preliminary tests showed that none of the listed compounds were inhibitory in the micromolar range. However, further testing of salicylamide and 4-aminosalicylic acid showed the MIC to be approximately 10 mM as shown in Fig. 3. This concentration is unlikely to be reached systemically, however topical application of microcrystalline 4-aminosalicylic acid potentially would provide a localized therapeutic dosage for wound infections. The investigation of the therapeutic potential of 4-aminosalicylic acid and efforts to identify additional siderophore synthesis inhibitors will continue during the second year of this grant.

The action of these inhibitors will be further confirmed by testing their effect on the production of DHBA and acinetobactin using the Arnow and CAS colorimetric tests, RP-HPLC and bioassays we have used to collect the data presented in Table 1. Compounds showing inhibitory effects using bacteriological media and laboratory conditions will be further tested with the *Galleria mellonella* experimental infection model as we recently described for the functional analysis of the acinetobactin-mediated system (7). In addition, we will explore the possibility of testing chemical compounds that inhibit specific siderophore biosynthetic steps other than DHBA biosynthesis (11) that may affect the iron uptake capacity of *A. baumannii* clinical strains. The ultimate goal of these studies is to determine whether potential siderophore biosynthesis inhibitors also display activity when tested in the animal models developed by Dr. Zurawski at WRAIR.

We also tested the effect of gallium salts such as gallium nitrate, the active component of the FDA-approved drug Ganite used in human medicine. Our initial work showed that the strain 19606 is sensitive to $Ga(NO_3)_3$ only when cultured under nutrient limiting conditions in the presence of DIP (3). This work also showed that the inhibitory effect of $Ga(NO_3)_3$ is independent of the production and utilization of acinetobactin as an iron chelator. However, a preliminary analysis of the MRSN strains listed in Table 1 showed that some but not all of them are sensitive to $Ga(NO_3)_3$ when cultured under nutrient- and iron-limiting conditions. Representative results are shown in Fig. 4 which shows that although strain 19606 grows in the presence of 100 μM DIP, it does not grow on M9 minimal medium agar plates containing 100 μM DIP and 100 μM $Ga(NO_3)_3$ (compare cognate sectors of plates in panels A and B of Fig. 4) as expected from our previous observations (3). The growth of *A. baumannii* M2, a clinical isolate we recently included in our collection because of its natural competence, also displayed a $Ga(NO_3)_3$ sensitivity response similar to that of 19606. However, the apparent growth of strains 17978, AYE and ACICU in the presence of 100 μM DIP and 100 μM $Ga(NO_3)_3$ does not match the observations recently published by Antunes *et al*. (12), who showed that these strains are sensitive when cultured in M9 minimal broth supplemented with up to 128 μM $Ga(NO_3)_3$. A similar observation was made when the MRSN strain 5075 was tested under the same experimental conditions. The differences with strains 17978, AYE and ACICU could be due to the use of agar plates rather than liquid cultures in microtiter plates, which although convenient to test a large number of strains may not favor bacterial growth because of poor aeration during incubation. We plan to address these issues by duplicating the conditions Antunes et *al.* used while testing all MRSN strains listed in Table 1.

We also tested the effect of Ga-protoporphyrin IX (Ga-PPIX), a non-iron metalloporphyrin that acts as an effective antimicrobial via hemin/hemoglobin utilization systems (13). Figure 5 shows that a disc containing 5 μmol, 10 μmol or 20 μmol Ga-PPIX deposited on the surface of LB agar plates seeded with 19606, 17978 or AYE bacteria produces a growth inhibition halo, the size of which is proportional to the concentration of Ga-PPIX present in the discs. In contrast to $Ga(NO_3)_3$, the growth inhibitory effect of Ga-PPIX is independent of the nutrient and iron content of the medium or the presence of DIP. Furthermore, the inhibitory response does not relate to the type of siderophore the strains use and/or produce. Screening of the MRSN strains listed in Table 1 using the same experimental conditions showed that all of them are sensitive to Ga-PPIX, including the MRSN strain 5075 (Fig. 5, bottom left panel). The growth inhibitory effect is also readily apparent with bacteria cultured in LB broth containing 31 μM or 61 μM Ga-PPIX (Fig. 5, bottom right panel). Taken together, these observations indicate that use of gallium containing compounds, particularly metalloporphyrin derivatives, could be a suitable and convenient approach to treat infections caused by strains resistant to multiple antibiotics. This hypothesis will be initially tested in our laboratory with the *G. mellonella* experimental infection model we used to demonstrate the virulence role of the acinetobactin-mediated iron acquisition system (7) and ultimately in animal models as proposed in Aim 2 of the research plan of this project.

Biofilm formation properties of the A. baumannii *MRSN strains*

It has been shown that biofilms play a critical role in the ability of bacterial pathogens to persist in medical settings and resist host defense factors and antibiotics. This seems to be a critical issue with *A. baumannii* and the infections it causes in wounded patients, such as injured military personnel. Therefore, we examined the capacity of the MRSN strains to form biofilms on glass and polystyrene, two abiotic surfaces normally found in medical settings and devices that could serve as reservoirs and facilitate the development of *A. baumannii* nosocomial infections. Table 2 shows that all tested strains formed biofilms on plastic when cultured statically at 37°C in LB broth. Furthermore, the strains have a tendency to form more biofilms when cultured in the presence of DIP, although some variations in biofilm formation among strains were observed. Panels A and B of Fig. 6 show some examples of the biofilms formed by the tested strains on polystyrene tubes under non-chelated and chelated conditions, respectively. Similar biofilm assays showed that the MRSN strains also adhere to and form biofilms on glass, although the capacity of the strains to interact with glass is more variable when compared with the response observed with plastic. Panels C and D of Fig. 6 show some examples of the biofilms formed on glass tubes under non-chelated and chelated conditions, respectively.

Since we reported that the ability of the strain 19606 to attach to and form biofilms on abiotic surfaces depends on the production of the CsuA/BABCDE usher-chaperone mediated pili (14), we determined the ability of the MRSN strains to produce CsuA/B, the major protein subunit of these pili. Table 2 shows that 13 out of 29 strains produce the CsuA/B protein, an observation that indicates that some of the MRSN strains may interact with abiotic surfaces and form biofilms using the 19606 CsuA/BABCDE usher-chaperone assembly system, while other isolates seem to use alternative cell systems that remain to be characterized. We also tested the motility of the MRSN strains on a semi-solid surface since cell motility could affect attachment and biofilm formation as we reported previously (15, 16). This approach showed that 13 out of 29 strains display motility on the surface of 0.3% agarose plates. Furthermore, the global analysis of the data presented in Table 2 indicates that there is not only a lack of correlation between the production of CsuA/B, and therefore the production of a particular type of pili, and the motility displayed by the different strains, but also a lack of correlation between their ability to form biofilms on different abiotic surfaces and their motility and CsuA/B production phenotypes. Taken together, these observations, which are in line with our recent report showing the same lack of correlation among a different set of clinical isolates (17), indicate that different *A. baumannii* isolates could use different cellular mechanisms to interact with and persist in medical environments and the human host, a potential challenge for the development of a universal biofilm inhibitor. However, additional studies using alternative approaches, such as testing biofilm formation on abiotic surfaces coated with relevant human/animal proteins that could play a role in this process or biotic surfaces provided by human cells, should be conducted before drawing final conclusions. This is an objective that will be pursued during the first quarter of the second year.

Selection of the A. baumannii *MRSN strain 5075 as the working model*
Considering all the information we have already collected and preliminary observations obtained by Dr. Daniel Zurawski with this strain, we decided to use isolate 5075 (AB5075) as the model strain to further test the role iron acquisition and biofilm formation functions play in the virulence of this strain. In addition, Dr. Zurawski has preliminary data showing that strain AB5075 is virulent in an animal experimental infection model. Preliminary data collected in our laboratory indicate that this strain, which grows under iron limitation and forms biofilms on plastic and glass (Tables 1 and 2, Fig. 6) and displays sensitivity to Ga-containing derivatives (Fig. 5), is also virulent when tested using the A549 human epithelial tissue culture (data not shown) and the *G. mellonella* infection models, which we used to test the role of the acinetobactin-mediated system in the virulence of *A. baumannii* 19606 (7). Following injection with 10^5 *A. baumannii* AB5075 cells larvae were assayed for survival. As shown in Fig. 7, infected larvae had significantly reduced ($P < 0.0001$) survival when compared to larvae either not injected or injected with sterile phosphate-buffered (PBS) saline solution. AB5075 killed 58% (29 or 50) of larvae by 120 h post infection, values similar to those we previously reported for strain 19606 (7). There was 80% (40 of 50) and 88% (44 of 50) survival of PBS injected and non-injected larvae, respectively. These results indicate AB5075 is virulent in the *G. mellonella* model. Equally important is Dr. Zurawski's observation that strain 5075 is amenable to random insertion mutagenesis using transposable elements as described in the following section. Thus, this strain is suitable for the *in vitro*, *ex vivo* and *in vivo* infection studies proposed to test the role of iron acquisition and biofilm formation functions expressed by *A. baumannii* and the identification of potential inhibitors that could be used to treat wound infections caused by multi-drug resistant strains.

Subaim 1.4. Generate isogenic derivatives using random approaches
Electroporation of AB5075 electrocompetent cells with transposable elements coding for resistance to hygromycin or tetracycline resulted in the isolation of a collection of insertion derivatives, some of which were analyzed by automated DNA sequencing. The transposable elements were generated by cloning the tetracycline or hygromycin resistance genes into the pMOD plasmid (Epicentre), which was mixed with purified Tn*5* transposase and electroporated into electrocompetent bacteria. It is important to note that pMOD is a suicide vector when introduced into *A. baumannii* cells (18). This work is being done in collaboration with Dr. Colin Manoil (University of Washington Genome Sciences, Seattle, WA), who is an expert in generating and sequencing bacterial mutant libraries using high-throughput approaches. Preliminary genetic and functional analyses of some AB5075 insertion mutants produced by Drs. Zurawski and Manoil showed the generation of

derivatives with single insertions in a wide range of genes coding for different functions including iron utilization (siderophore transport and biosynthesis) as well as functions potentially involved in surface attachment and biofilm formation (adhesins and cell-surface-located pili). These results, which are critical for the progress of this project, will allow us to generate appropriate AB5075 isogenic derivatives affected in key virulence functions, which could be tested in the animal models proposed in this project. These derivatives together with the isogenic AB5075 wild-type parent strain will also be useful tools to test the efficacy of iron utilization and/or biofilm formation inhibitors. The detailed genetic and functional analyses of these mutants will start during the second year of this award as proposed in Aim 2 of this project.

Subaim 1.3. Generate luminescent derivatives

Some of the proposed virulence studies using experimental infection models will require the availability of *A. baumannii* easily detected by fluorescence or luminescence. We decided to pursue the second option since luminescent bacterial derivatives will be suitable for some of the *in vivo* virulence studies proposed in this project. For this purpose, we are in the process of generating a plasmid-based reporter system in which the *luxABCDE* genes are cloned into the *E. coli*-*A. baumannii* shuttle plasmid vector pWH1266. We are generating derivatives of this cloning vector coding for tetracycline or hygromycin resistance to be introduced either into the AB5075 parental strain or the AB5075 insertion mutants generated with transposons coding for hygromycin or tetracycline resistance, respectively. The presence of the pWH1266 expressing the *luxABCDE* operon within bacterial cells will facilitate the location and tracking of bacteria during the infection process in animals and organs with the Xenogen IVIS 100 imaging system available at WRAIR as proposed in Aim 2 of this project. We also plan to use the mini-Tn*7* system recently adapted to *A. baumannii* (19) to generate AB5075 *luxABCDE* chromosomal insertion derivatives, which could be more stable than plasmid-based reporter constructs.

Key Research Accomplishments

- Identification of a distinct set of MRSN *A. baumannii* strains isolated from wounded military personnel.
- Demonstration of the ability of selected *A. baumannii* strains to grow under iron limitation and produce/secrete iron-regulated siderophore compounds, most of which derive from dihydroxybenzoic acid (DHBA).
- Potential sensitivity of MRSN strains to DHBA inhibitors that could affect active iron acquisition under limiting conditions.
- Apparent growth inhibitory action of gallium-containing compounds, particularly by Ga-protoporphyrin, independently of the nutrient and iron content of the culture media.
- Selection of a working model strain, AB5075, which forms biofilms, grows under iron limitation, produces the siderophore acinetobactin and is genetically amenable.
- Evidence of the AB5075 virulence using the *G. mellonella* experimental infection model, which could be used as a convenient tool to study the role of iron acquisition and biofilm formation functions expressed by this pathogen and screen/test the efficacy of potential inhibitors affecting these critical virulence functions.
- Suitability of AB5075 to critical genetic manipulations, such as random mutagenesis and genetic complementation using cloned wild-type genes.
- Generation of AB5075 insertion mutant libraries that will be critical for conducting proposed functional and virulence studies.

Reportable Outcomes

Presentations

Actis, L. A. Shedding light on *Acinetobacter baumannii* virulence. Annual meeting, Ohio Branch of the American Society for Microbiology, Ashland University, Ashland, Ohio, April 2013. Results regarding the iron uptake phenotype of *A. baumannii* MRSN strains and the effect of iron uptake inhibitors were presented and discussed in the meeting.

Conclusion

Work done during the first year of the award resulted in the identification and characterization of a set of *A. baumannii* strains isolated from wounded military personnel with regard to their capacity to grow under iron limiting conditions, produce iron chelators – siderophores – and form biofilms on abiotic surfaces normally found in medical settings. Considering the variations in the different types of siderophores these strains may produce and/or use, using iron acquisition as a therapeutic target could be a challenge. Nevertheless, the data collected during this time showed that gallium-containing derivatives, particularly Ga-protoporphyrin IX, could be a useful therapeutic agent since all tested strains were sensitive independently of the nutrient and iron content of the media or the iron uptake capacity of the tested strains. Furthermore, this compound showed no toxicity when used by other investigators to treat animal infections caused by different bacterial pathogens (20). Overall, these are encouraging observations that will be tested in the animal models proposed in this project and promote future basic studies since the mechanisms by which Ga-PPIX inhibit *A. baumannii* growth are unknown. Equally relevant is the fact that we selected the MRSN AB5075 as a model strain for more extensive studies because of its virulence, when tested using different experimental infection models, and amenability to genetic manipulation, including genetic complementation and random/site directed mutagenesis. Meeting these two critical conditions will allow us to conduct the virulence and inhibition studies proposed in this project. Furthermore, the availability of an AB5075 insertion library will allow members of the scientific community to study novel and relevant aspects of the virulence of *A. baumannii* and the host-pathogen interactions that play a role in the pathogenesis of the infections this microbial agent causes in wounded military personnel as well as civilian patients.

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Appendices

None.

Table 1. Iron acquisition properties of selected MRSN *A. baumannii* isolates.

Isolate	Site	PFGE[a]	MIC[b]	CAS assays[c]	Arnow assays[d]	BauA production[e]	*bauA* assays[f]	*basD* assays[g]	HPLC[h]
967	Blood	10	-	+	+	+	+	+	1
2828	Blood	4	-	+	+	+	+	+	1
3340	Blood	8	-	+	+	+	-	+	1
3560	Blood	1	+	+	+	+	-	+	4
3638	STS¶	4	-	+	+	+	+	+	1
3785	Blood	3	+	+	+	-	+	+	1
3806	*STS*	*4*	-	-	-	-	-	-	*1*
3927	Tibia	ND	-	+	+	+	+	+	3
4025	Femur	ND	+	+	+	+	+	+	1
4026	Fibula	ND	+	+	+	+	-	+	1
4027	Femur	ND	+	+	+	+	-	+	1
4052	War wound	23	+	+	+	+	-	+	5
4269	War wound	5	+	+	+	+	+	+	1
4448	War wound	34	+	+	+	+	+	+	1
4456	Tachasp£	27	+	+	+	+	+	+	4
4490	War wound	34	+	+	+	+	+	+	1
4498	Blood	3	-	+	+	-	-	+	3
4795	STS¶-Bone	6	-	+	+	+	-	+	1
4857	STS-Bone	12	+	+	+	+	-	+	4
4878	War wound	11	+	+	+	+	-	+	4
4932	Sputum	25	+	+	+	+	+	+	1
4957	STS-Bone	2	+	+	+	+	+	+	1
4991	War Wound	21	+	+	+	+	+	+	1
5001	Blood	2	+	+	+	+	+	+	1
5075*	Bone	ND	+	+	+	+	+	+	1
5197	STS	7	+	+	+	+	-	+	3
5256	Blood	1	-	+	+	+	+	+	1
5674	Blood	9	-	+	+	+	+	+	1
5711	Blood	2	+	+	+	+	+	+	1
19606§	Urine	NA	+	+	+	+	-	+	1
17978§	meningitis	NA	+	+	+	+	+	+	5
ACICU§	meningitis	NA	+	+	+	+	+	+	4
AYE§	Urine	NA	+	+	-	+	+	+	2

[a]Pulse field gel eletrophoresis (PFGE) type, ND, not determined, NA, not available. [b]Minimal inhibitory concentration (MIC) of DIP at 250 μM. (-), no growth; (+) growth after overnight incubation at 37°C. All strains grew in LB broth with 150 μM DIP. [c]Detection of siderophore production with CAS reagent; (+), positive reaction; (-), negative reaction. [d]Detection of catechols with the Arnow colorimetric reaction; (+), positive reaction; (-), negative reaction. [e]Detection of BauA in total lysates of bacteria cultured under iron chelation using anti-BauA antiserum; (+), positive reaction; (-), negative reaction. [f]Detection of acinetobactin or unrelated siderophore production using a 19606 t6 derivative that does not use acinetobactin because of a mutation in the *bauA* gene coding for the acinetobactin receptor; (+), positive reaction; (-), negative reaction. [g]Detection of acinetobactin or unrelated siderophore production using a 19606 s1 mutant that does not produce acinetobactin because of a mutation in the *basD* biosynthesis gene; (+), positive reaction; (-), negative reaction. [h]Detection of acinetobactin and DHBA by HPLC analysis of iron-restricted culture supernatants. The numbers represent the type of HPLC profile as defined in Fig. 1. ¶Severe trauma site. £Tracheal aspiration case. §Control strains. *MRSN model strain.

Table 2. Production of biofilms, CsuA/B protein and motility of selected MRSN *A. baumannii* isolates.

Strain	Plastic[a]		Glass[a]		Production of CsuA/B protein[b]	Motility on 0.3% agarose[c]
	LB	LB +DIP	LB	LB +DIP		
967	++	+	+	++	-	-
2828	++	++	++	++	-	-
3340	++	++	+	-	-	+
3560	++	++	+	-	-	-
3638	++	++	++	+++	-	-
3785	+++	++	-	+	+	-
3806	++	+++	+++	++	-	+
3927	++	++	-	+	-	+
4025	+++	++	+	+	+	+
4026	+++	+++	-	++	+	+
4027	+++	++	+	-	+	+
4052	++	+++	+++	-	+	-
4269	++	+++	+	++	+	-
4448	+	+	+	+	-	+
4456	+	++	+	+	-	+
4490	+	+	++	-	-	+
4498	+++	++	+	++	+	+
4795	++	++	-	-	-	-
4857	+	++	+++	+	-	-
4878	++	+++	+	+	+	-
4932	++	+++	+++	+	+	-
4957	++	+++	+++	+++	+	+
4991	+	++	++	+	-	-
5001	++	+++	+++	+++	+	-
5075	+	-	-	-	-	+
5197	+	+	+	+	-	-
5256	++	+++	+++	++	-	+
5674	+	++	+	++	+	-
5711	++	++	++	++	+	-
19606[§]	+++	+++	+	+++	+	-
17978[§]	++	-	+	-	-	+
ACICU[§]	+	++	+	-	-	-
AYE[§]	+	+	+	+	-	+
19606-(20)BfmR[§]	-	-	-	-	-	-

[a]Biofilm formation was classified as negative (-), weak (+), moderate (++) and strong (+++) when compared with biofilms formed by the 19606 and 19606-BfmR positive and negative control strains, respectively. [b]Detection of the CsuA/B pilin protein in total bacterial cell lysates using anti-CsuA/B antiserum; (+), positive reaction; (-), negative reaction. [c]Detection of bacterial motility on the surface of 0.3% agarose plates after overnight incubation at 37°C. [§]Control strains. Strain 19606-BfmR is a mutant that does not produce pili and does not make biofilms on abiotic surfaces; it was used as a negative control.

Fig. 1. Chromatographic profiles of *A. baumannii* iron-restricted culture supernatants analyzed by HPLC. Ab, acinetobactin; DHBA, dihydroxybenzoic acid.

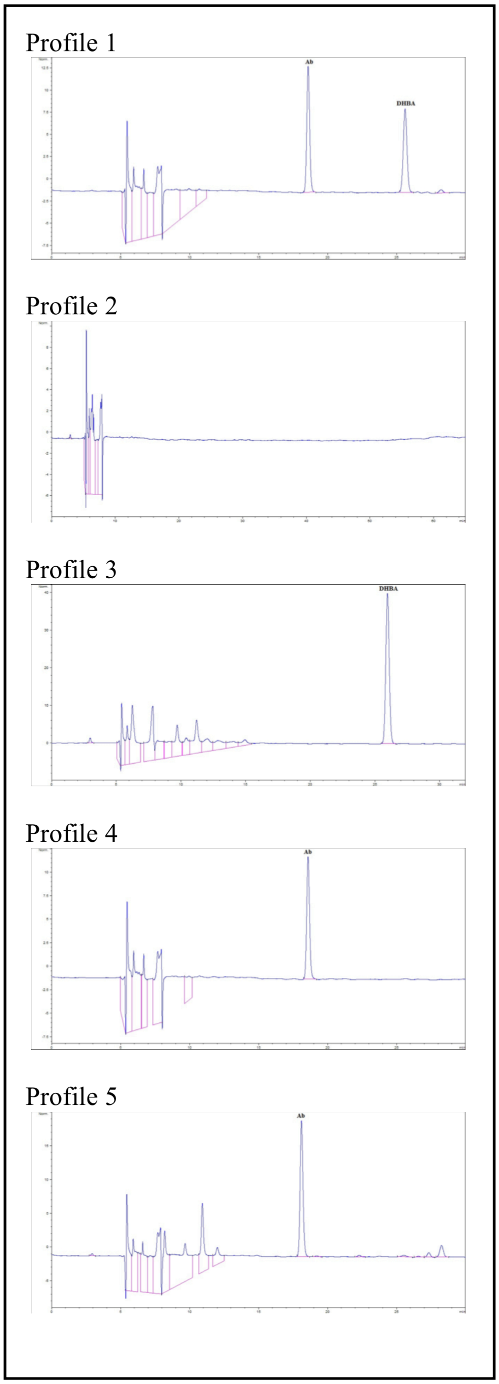

Fig. 2. Reactivity of the anti-19606/17978 antibodies with total membranes isolated from four different *A. baumannii* strains. (A) Western blot analysis using HRP-labeled protein A and chemiluminescence. (B) Flow cytometry of whole bacterial cells using FITC-labeled anti-rabbit secondary antibodies.

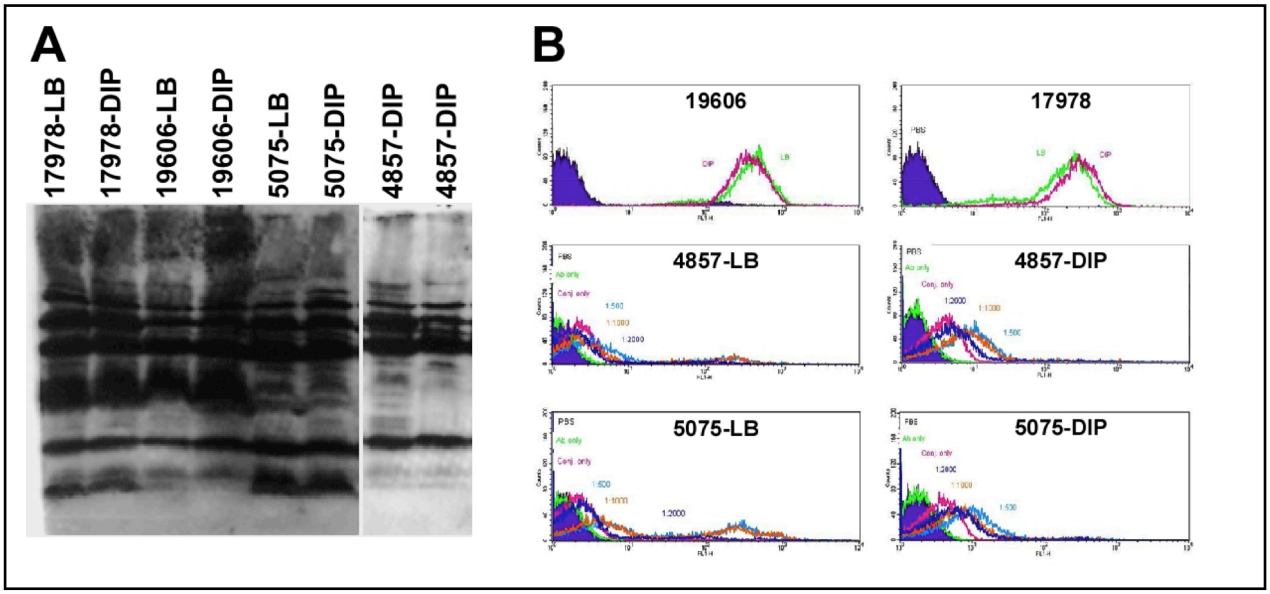

Fig. 3. Effect of salicylate derivatives on the growth of *A. baumannii* AB5075. Bacteria were grown in M9 minimal medium supplemented with 100 μM DIP and either salicylamide or 4-aminosalicylic acid at 37°C shaking at 200 rpm for 24 h. Bacterial growth was determined spectrophotometrically at OD_{600}.

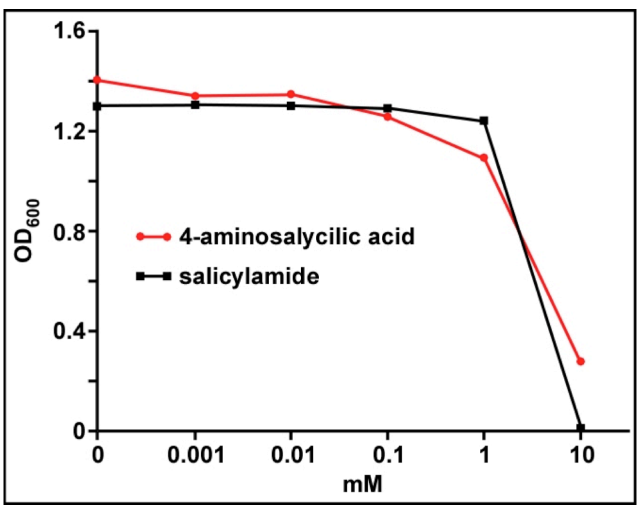

Fig. 4. Effect of $Ga(NO_3)_3$ on *A. baumannii* growth. Strains were streaked on the surface of M9 minimal medium agar plates containing 100 μM DIP (A) or 100 μM DIP and 100 μM $Ga(NO_3)_3$ (B). Bacterial growth was recorded after overnight incubation at 37°C.

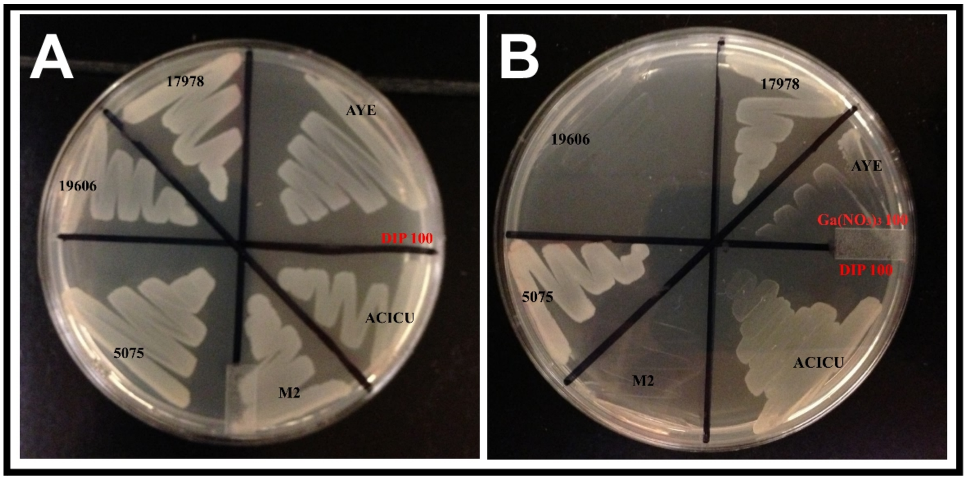

Fig.5. Effect of Ga-PPIX on the growth of *A. baumannii* strains cultured on LB agar plates or LB broth. Bacterial cells were seeded on the surface of LB agar plates before sterile discs impregnated with 10 μl of a sterile aqueous solution containing 5 μmol, 10 μmol or 20 μmol Ga-PPIX were deposited on the surface of the plates. The effect of Ga-PPIX on the growth of AB5075 was also tested in LB broth cultures containing either no Ga-PPIX (0) or supplemented with 31 μM or 62 μM Ga-PPIX. Bacterial growth was recorded after overnight incubation at 37°C. Broth cultures were incubated in a shaking incubator set at 200 rpm.

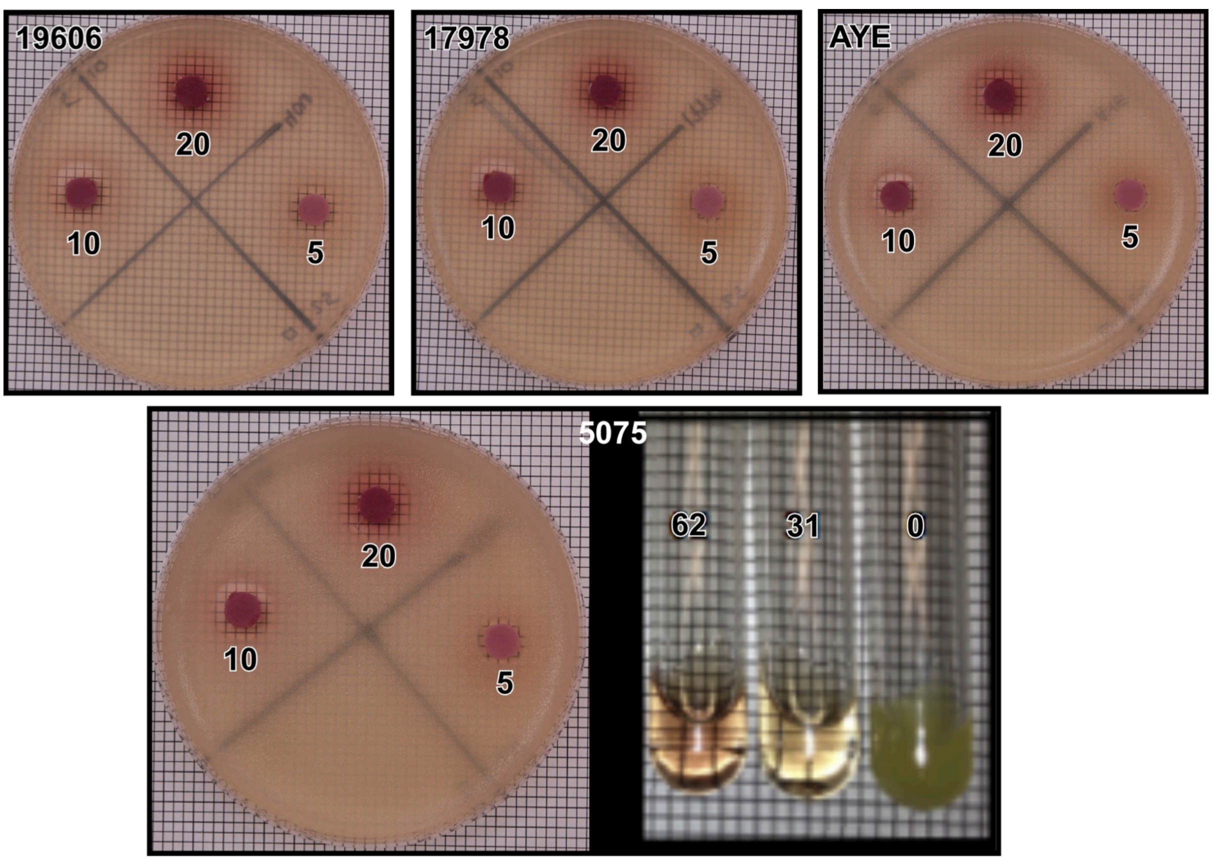

Fig. 6. Biofilms formed by *A. baumannii* strains. Bacteria were cultured in plastic (A and B) or glass (C and D) tubes using LB broth with no supplementation (A and C) or containing 100 μM DIP (B and D). Cultures were incubated at 37°C statically overnight and biofilms were detected by crystal violet straining after removing the culture supernatants and washing the tubes with distilled water.

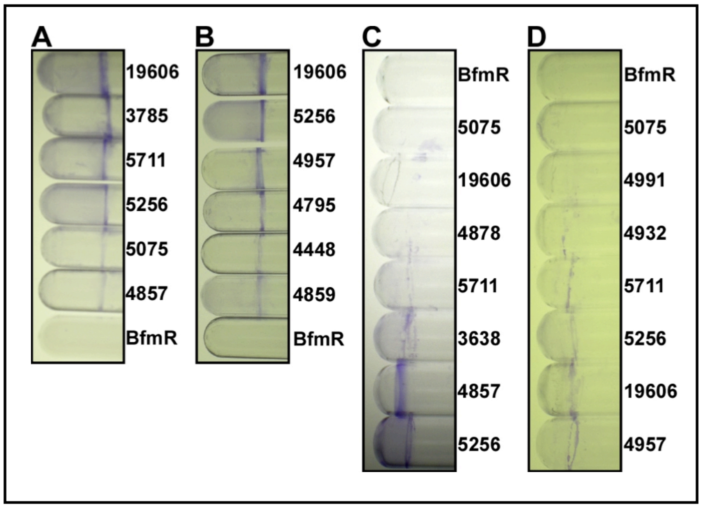

Fig. 7. Virulence of strain AB5075. The virulence of this strain was tested by injecting *G. mellonella* larvae with 1 x 10^5 bacteria and incubated at 37°C in darkness. Moth death was determined daily for five days. Caterpillars not injected or injected with comparable volumes of sterile PBS were used as negative controls. Experiments were repeated five times using 10 larvae per experimental group and the survival curves were plotted using the Kaplan-Meier method (21). *P* values <0.05 were considered statistically significant for the log-rank test of survival curves (Graphpad Software Inc., La Jolla, Ca).